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A simple method for site-directed mutagenesis using the polymerase chain reaction

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ABSTRACT

We have developed a general and simple method for directing specific sequence changes in a plasmid using primed amplification by the polymerase chain reaction (PCR). The method is based on the amplification of the entire plasmid using primers that include the desired changes. The method is rapid, simple in its execution, and requires only minute amounts of plasmid template DNA. It is significant that there are no special requirements for appropriately placed restriction sites in the sequence to be manipulated. In our system the yield of transformants was high and the fraction of them harboring plasmids with only the desired change was consistently about 80%. The generality of the method should make it useful for the direct alteration of most cloned genes. The only limitation may be the total length of the plasmid to be manipulated. During the study we found that the Taq DNA polymerase used for PCR adds on a single extra base (usually an A) at the end of a large fraction of the newly synthesized chains. These had to be removed by the Klenow fragment of DNA polymerase to insure restoration of the gene sequence.

INTRODUCTION

Site-directed mutagenesis is a powerful method that is commonly used in several areas of molecular biology and biochemistry. Early methods for site-directed mutagenesis using single-stranded plasmids (or segments of plasmids) gave low efficiencies of obtaining the desired mutant sequences (1). Improvements which employ selection against the wild-type sequence (2,3) are costly in time and effort taken to isolate mutants. The development of the polymerase chain reaction (PCR) (4,5,6) however, has led to new approaches to site-directed mutagenesis (7,8,9). These methods use primers which contain one or several bases which differ from the wild-type sequence, which, after PCR, are then incorporated into the PCR product. This altered sequence is then cleaved with restriction enzymes and inserted in place of the wild-type sequence. This is a relatively time-consuming approach, and relies on the presence of conveniently located restriction sites flanking the wild-type sequence being mutated.

We present here a rapid approach to PCR based site-directed mutagenesis, using an adaptation of inverse PCR (10,11), whereby an entire circular plasmid is amplified and a mutation inserted anywhere in the plasmid with no requirement for convenient restriction enzyme sites. In this technique, the initial rounds of amplification are directed by two primers located 'back-to-back' on the opposing DNA strands (see Figure 1.). One primer contains one or a few mismatches which will generate the site-directed mutation. The first cycle of PCR generates linear plasmid molecules, from one or both circular template strands. Subsequent rounds of PCR further amplify the linear plasmid sequence generated by the first PCR cycle, which is then isolated, ligated and used for transformation. This method

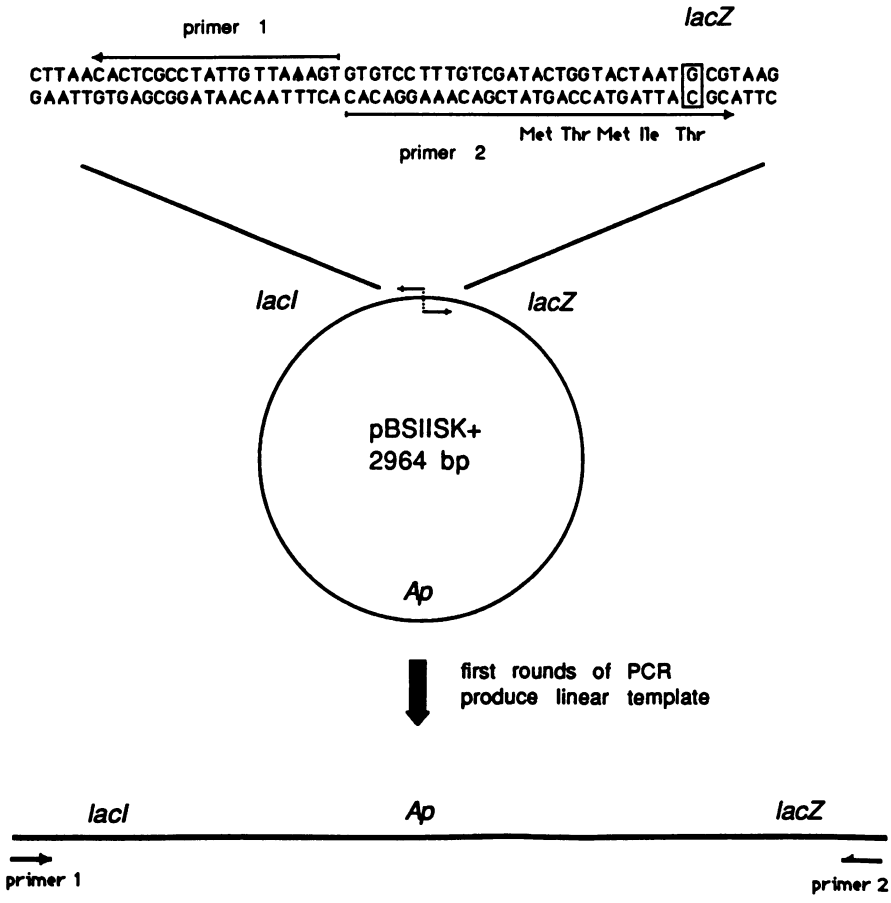

Figure 1. Schematic of the inverse PCR procedure. The expanded sequence shows the 'back-to-back' positions of the two primers with respect to each other and the site of the introduced mutation (deletion of the boxed base pair) in the coding sequence of the *lacZ* alpha peptide.

has the advantage of introducing mutations at any desired site, independent of whether restriction sites are present in the surrounding sequence. In addition, the concentration of the initial wild-type template after generation of the mutant plasmid PCR product is negligible so that very few wild-type clones would be expected. We used the plasmid pBluescript II SK^+ as a model system, since it offers a convenient visual detection system to monitor introduced changes in the alpha peptide sequence of *lacZ*. (Fig. 1.) Figure 2 shows the method in schematic form. The mutation we chose to introduce into the alpha peptide gene resulted in a frameshift (single base-pair deletion).

MATERIALS AND METHODS

Primer synthesis

Synthetic oligonucleotide primers complementary to segments of the *lacZ* operator region and alpha peptide coding sequence were prepared on an Applied Biosystems 381A DNA

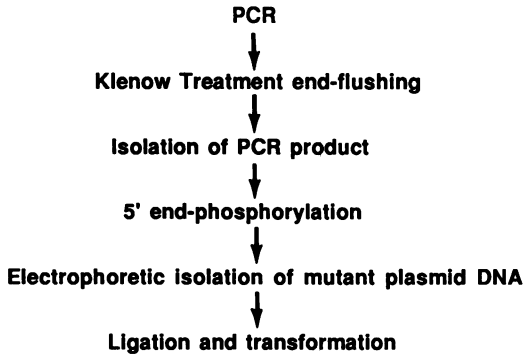

Figure 2. Schematic summary of the steps involved in the construction of mutants.

synthesizer. Primers were constructed so as to lie 'back to back' on the duplex, with 5′ ends apposing and 3′ ends oriented for extension in opposite orientations around the plasmid circle (see Fig. 1).

A pair of primers with sequences exactly complementary to the pBluescript II SK$^+$ sequence were synthesized as a control to confirm that the amplification process was able to faithfully regenerate the original sequence (designated wild-type primers, sequences 5′ TGAAATTGTTATCCGCTCAC 3′ and 5′CACAGGAAACAGCTATGACCATGATTACGC 3′). In the experimental primer set, (designated mutant primers, sequences 5′ TGAAATTGTTATCCGCTCAC 3′ and 5′ CACAGGAAACAGCTATGACCATGATTAGC 3′), one primer has a deletion corresponding to the 14th nucleotide in the coding region of the *lac*Z alpha peptide, designed to eliminate B-galactosidase activity.

Template preparation

The DNA used as template for the PCR reaction was extracted from *E.coli* strain JM109 transformed with pBluescript II SK$^+$ (Stratagene stock preparation). Plasmid DNA was isolated by a standard alkaline lysis mini-prep method (12).

PCR conditions

Template DNA (10 fmol) and primer sets (1 μM each) were incubated in a Perkin Elmer Cetus Thermal Cycler in 100 μl reaction volumes containing 100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.2 mM of each dNTP and 2 units Taq polymerase (Perkin Elmer-Cetus)(6). The amplification proceeded through a cycle of denaturation at 94° C (1 min), annealing at 45° C (1 min) and primer extension at 72° C (12 min) for a total of 25 cycles. Shorter extension times gave poor results for full length amplification.

Klenow treatment end-flushing

Ten microliter portions of the samples generated by PCR were analysed on a vertical agarose gel (1.2%) to determine the efficiency of the amplification. The remainder (90 μl) from 3−4 samples was pooled and the four dNTP'S were added to a final concentration of 250 μM each. Klenow fragment of *E.coli* polymerase I was added (20units) and the reaction mix was incubated at 37° C for 30 min. This step was necessary to avoid added basepairs at the ligation point (see results).

DNA purification and 5′ end-phosphorylation

Double stranded DNA was isolated from the PCR reaction mix using the standard protocol for Geneclean DNA purification (Geneclean ™, BIO 101 Inc.) and phosphorylated at the

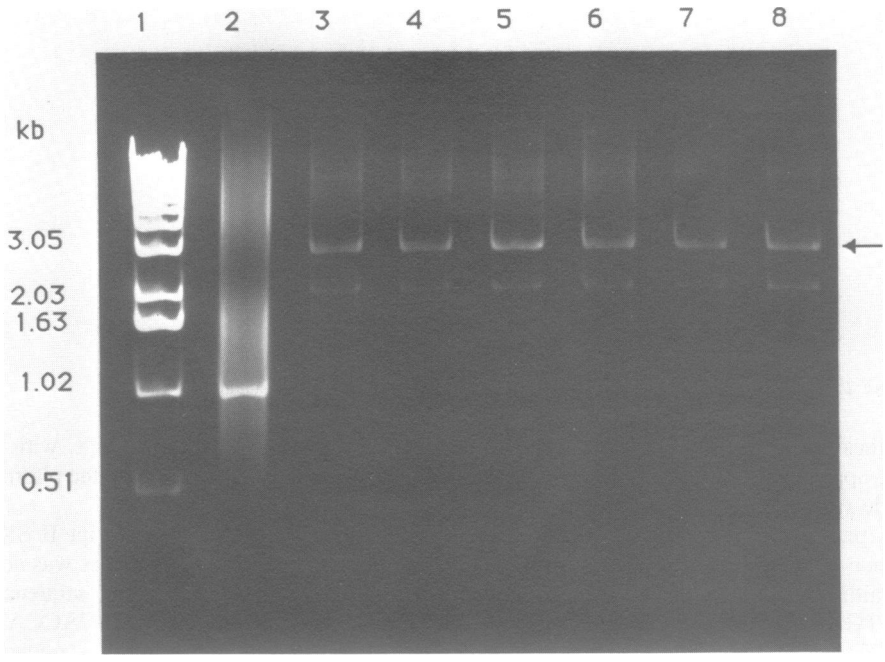

Figure 3. Agarose gel electrophoresis of PCR amplification products (10μl of 100μl reaction mixes) using wild-type primers to regenerate unchanged pBluescript (lanes 3–5) and mutant primers to amplify plasmid DNA containing a frame-shift within the alpha-peptide of the *lacZ* gene (lanes 6–8). Lane 2 shows an equivalent amount of a control product, where the same amount of template DNA was used to amplify a 1kb fragment. PCR product sizes are assessed against 1kb DNA Ladder(BRL) (lane 1).

5′ end by incubation with 15–20 units of T4 polynucleotide kinase (Amersham) in a 25 μl reaction volume containing 66 mM Tris-HCl, pH 7.6, 1 mM ATP, 1 mM spermidine-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.2 mg/ml BSA (13).

Isolation and ligation of the plasmid DNA

The phosphorylated DNA samples generated from the PCR reactions were then subjected to electrophoresis in a low melting temperature agarose gel (1% Seaplaque agarose). The ethidium bromide-stained bands of DNA corresponding to a size of 3kb were excised, heated to 65°C, and 6 μl samples were added to standard blunt-end ligation buffer and T4 DNA ligase (approximately 12 units, BRL) in a final volume of 10 μl. The ligation mixes were incubated at 25°C for time periods ranging from 8 hours to overnight.

Transformation

The ligation mix was reheated to 65°C and 250 μl of freshly prepared competent cells (strain NM522) were transformed by a standard procedure(14).

RESULTS

Plasmid amplification

To test the method, we used a small (2964 bp) cloning vector carrying the alpha peptide gene segment of B-galactosidase, a colorimetric indicator gene. We modified the usual PCR protocol as shown in Materials and Methods. Successful PCR amplification of the

Table 1. Phenotypic and sequence analysis of the results of the directed mutagenesis protocol using wild-type or mutant primers. OK indicates that the sequence observed was the correct pBluescript sequence.

Primers	White colonies	Blue colonies
Wild type	79 (7%)	1070 (93%)
Mutant	1326 (97%)	42 (3%)
Sequenced:	29	7
Mutation site	29 mutant	5 wt.
		2 +G
Ligation site	24 OK	6 OK
	4 del.,	1 del.,
	1 +A	

entire pBluescript II SK$^+$ plasmid is shown in Figure 3. The other bands in each lane were not characterized further, and undoubtedly resulted from the low temperature of primer annealing during PCR. The second lane of the gel shows amplification of a 1kb segment (using a different primer pair) from the same plasmid as a control, giving some indication of the relative efficiency of amplification.

The protocol used for the experiments described here is outlined in the diagram in the introduction with the details provided in the Materials and Methods. In all cases it is necessary to begin with only minute amounts of plasmid DNA prepared by a frequently used rapid 'mini-prep' procedure.

Colony formation

The re-circularized products of the amplified plasmids were transformed by a standard protocol with good yield of colonies. The control experiment, using wild-type primers to regenerate the original pBluescript plasmid generated 1149 colonies, 1070 (93%) of which were blue, suggesting an unaltered *lacZ* sequence. Mutant primer amplification generated 1368 colonies, 1326 (97%) of which were white, the preliminary diagnostic criterion for successful insertion of the frameshift mutation into the *lacZ* alpha peptide. (See Table 1).

Sequence data

The nucleotide sequence of the region shown in the inset of Figure 1 was examined for 36 colonies generated by using the mutant primers. Both white (29 colonies) and blue (7 colonies) colonies were checked to assess the spectrum of alterations in nucleotide sequence which led to each phenotype. It was expected that only alterations introduced in the primer would lead to a phenotypic change in the colony, since the point of ligation of the plasmid was designed to lie outside of the coding region of the alpha peptide of *lacZ* in order that changes in sequence at the ligation point could easily be distinguished from changes at the intended position of mutation. Results of the sequence analysis are shown in Table 1. Of the 36 plasmid sequences examined, 30 (83%) restored the wild-type sequence at the ligation point. Five out of the other six plasmids had small (1−8 bp) deletions at the site of the ligation and one had an additional adenine (A) inserted at the ligation point. One possible explanation of the small deletions is that they are the result of incorporation into the PCR product of a primer which was not synthesized to full length, as the primers used were not purified after synthesis.

In experiments without the Klenow fragment end-flushing step, the insertion of an extra nucleotide at the ligation position was seen to occur at a much higher frequency; 73% of the 24 sequences examined (data not shown). It is interesting that the extra base pair

was an A:T in all cases but one. This added base-pair at the ligation point was most likely due to the non-templated addition of a base to the ends of the synthesized DNA by the Taq polymerase (15). In preliminary experiments, using a different primer-template system, we found that a large fraction of the PCR products had a single extra base present that was removed when the double-stranded product was treated with Klenow fragment as described in the present protocol (data not shown).

All 29 white colonies produced using the mutant primers had the intended mutant sequence correctly inserted into the early coding region of the *lacZ* alpha peptide. The blue colonies had two sequence variations; five of the seven were wild-type sequence, of unknown origin, while the other two had an additional nucleotide inserted in the primer region, restoring the reading-frame to that of wild-type. This suggests a mutation made by slipped mispairing by the *E.coli* DNA polymerase, since a run of two guanine nucleotides (G's) was expanded to three.

DISCUSSION

In this paper, we demonstrate that it is possible to generate linear fragments of at least 3 kb in length, constituting an entire plasmid, using intact circular plasmid DNA as a template. We have further demonstrated that it is a simple matter then to site-direct changes in the sequence. The level of efficiency of amplification is less than that observed when smaller fragments are amplified, however sufficient DNA is synthesized for subsequent ligation and transformation procedures. Based on the data obtained from analysis of 29 candidate colonies, insertion of the desired mutation and exact reconstitution of the plasmid, occurs at a frequency of 82%. The desired mutation is inserted at a frequency of about 95%, so that improvements in procedures to reconstitute the ligation site might enhance the overall efficiency.

This procedure is consistently efficient, rapid (mutant colonies can be generated in two to three days) and generates substantial numbers of transformants. An additional advantage is that it does not require highly purified DNA as the template for the PCR reaction and there are no special requirements for the plasmid preparation. It should also be possible to use plasmids liberated from small numbers of bacterial cells taken directly from plates, which would further shorten the procedure. We suspect that open circular plasmid provides the best template for inverse PCR, since it was observed that template derived from minipreps was actually a better substrate for PCR amplification than was highly purified, supercoiled plasmid (data not shown).

The relatively long PCR extension times we used for efficient elongation of the 3 kb DNA fragment (12 min.) could have affected the frequency of non-template-directed nucleotide addition at the 3′ ends of the molecules. Removal of these nucleotides can be effected by a brief treatment with Klenow treatment of the PCR reaction under the conditions described in the Materials and Methods section.

The mutations generated in this study were localized to the *lacZ* gene to permit a simple visual assay of successful mutagenesis. The independence of this method on the proximity of unique restriction sites makes it a convenient and rapid method whereby mutations may be introduced at any site within any circular DNA molecule at an extremely high mutant to wild-type ratio. At the present time, it is conceivable that plasmids up to 10kb in length could be used in our system (16).

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